STAPHYLOXANTHIN MUTANTS OF STAPHYLOCOCCUS AUREUS AND THEIR RESPONSE TO ANTIMICROBIALS

A Thesis

Presented to the Honors Program of

Angelo State University

In Partial Fulfillment of the

Requirements for Highest University Honors

BACHELOR OF SCIENCE

by

WILLIAM HUNTER MIEARS

May 2018

Major: Biology

STAPHYLOXANTHIN MUTANTS OF STAPHYLOCOCCUS AUREUS AND THEIR RESPONSE TO ANTIMICROBIALS

by

WILLIAM HUNTER MIEARS

APPROVED:

Dr. Crosby W. Jones, Chair
Professor of Biology

Dr. Laurel Fohn
Assistant Professor of Biology

April 27, 2018
Date Successfully Defended and Approved by Advisory Committee

APPROVED:

Dr. Shirley M. Eoff
Director, Honors Program

May 11, 2018

ACKNOWLEDGEMENTS

I would like to acknowledge the Tri-Beta Biological Honors Society for awarding me an undergraduate research grant to fund my project. This project would not have been possible without the support from countless people. I would especially like to thank Dr. Crosby Jones for serving as my research advisor for three years and also as the chair of my thesis committee. He sparked my interest in microbiology and continually provided new information to help me grow my knowledge and gain an appreciation for this field during my time at Angelo State University. This project would not have been possible without his continuous support, time investment, and knowledge. I would also like to thank Dr. Laurel Fohn for serving as a member of my thesis committee and keeping me organized during the writing process and Dr. Nicholas Negovetich for assisting me in the analysis and interpretation of my data. Additionally, I would like to thank and acknowledge the Honors Program Director, Dr. Shirley Eoff, for constantly pushing me to succeed, to present my research, and for allowing me the opportunity to complete an Honors undergraduate thesis, and for serving as a role model and sounding board during my time at Angelo State University. I would also like to acknowledge Angelo State University for providing me with the numerous opportunities, scholarships, and faculty that have helped to shape who I have become today. Finally, I would like to thank the countless friends and family that helped to support and encourage me through my undergraduate career at Angelo State University.

ABSTRACT

Staphyloxanthin is a golden pigment that is produced by the bacterium, *Staphylococcus aureus*. This pigment is a virulence factor for the bacterium and is hypothesized to have the dual function of acting as an antioxidant to protect against action of oxidizing agents, a common form of immune response in the human body, and stabilizing the cell membrane, much like cholesterol does in human cells. In this experiment, mutants with little or no staphyloxanthin were successfully created using UV light. These mutants were then tested against various agents, including oxidants, cell membrane active antibiotics, and *Pseudomonas aeruginosa* exoproducts, to determine if there was a difference in bacterial response between the mutants and the wild type, or non-mutated, bacterium that still produced the staphyloxanthin pigment. I hypothesized that the lack of staphyloxanthin would cause the mutant bacteria to be more drastically affected by the antibacterial agents. This was tested using a standard Kirby-Bauer method where the test compounds are impregnated into standard filter paper discs and tested against lawns of bacteria. Zones of inhibition were measured after 48 hours. Among other data recorded was the influence on pigment production of the various test compounds. Results indicate that differentially pigmented mutants of *S. aureus* differ in their response to cell membrane active antibiotics and *P. aeruginosa* exoproducts.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

INTRODUCTION

General Information:

Staphylococcus aureus is a gram-positive bacterium that is endemic, or native, to the human body. Despite its reputation as a common pathogen, most strains of *S. aureus* are not harmful when relegated to their normal habitat on the body (1). However, if the bacterium is able to enter the blood stream or an open wound, this can be one of the most problematic bacterial infections to overcome. Not only can *Staph aureus* directly infect humans, it also impacts human health and economics because of its infection of livestock and presence in the food industry. Much of the damage *S. aureus* causes is due to its virulence, adaptivity (including acquisition of antibiotic resistance), and interactions with other bacteria (2).

S. aureus and *Pseudomonas aeruginosa*:

S. aureus is present in many cases of superinfection in the human body. In a superinfection, two or more bacterial species inhabit the same wound or area and both contribute to the symptoms exhibited by the patient. These infections are extremely difficult to treat and can occur in a variety of places in the human body (3). One of the most common cohabitants in *S. aureus* superinfections is the bacterium, *Pseudomonas aeruginosa*.

P. aeruginosa is an opportunistic pathogen that is not highly infectious by itself (3). However, when present in a polymicrobial community, such as in superinfections, both *P. aeruginosa* and *S. aureus* become more pathogenic than they are when isolated (3). Common examples of superinfections involving these bacteria are infections found in cystic fibrosis

(CF) patients, burn infections, and diabetic ulcers (3). When both of these bacteria are present, *P. aeruginosa* secretes a host of exoproducts that interfere with the growth, metabolism, and homeostasis of *S. aureus*, making it more susceptible to both antibiotic treatment and host immune defense (3). Two of these *P. aeruginosa* products, 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) and pyocyanin, was focused on throughout this study.

HQNO belongs to a class of molecules known as 2-alkyl-4-(1H)-quinolones (AQ) (4). This particular AQ is a cytochrome B inhibitor (4). In the *S. aureus* cell, cytochrome B acts in the cellular membrane to assemble the enzyme, nitrate reductase (5,6). This enzyme allows the bacteria to reduce nitrogen and use it as an electron donor (5,6). This means that the ability of *S. aureus* to use the electron transport chain (ETC) to generate ATP is severely inhibited when HQNO is present (3). This exoproduct is one of the main mechanisms for allowing *P. aeruginosa* to overtake *S. aureus* in superinfections. It is because of these properties of HQNO that it was chosen as one of the antibacterial agents in this study.

Pyocyanin is a blue-green pigment that is one of the characteristic exoproducts secreted by *P. aeruginosa*. Like HQNO, pyocyanin also acts to inhibit the respiration of *S. aureus*, eliminating its ability to use the ETC for ATP production (7). This forces the *S. aureus* to adopt a phenotype known as a small-colony variant phenotype (SCV) (7). These SCV colonies are then susceptible to pyocyanin dependent reactive oxygen species (ROS) that are extremely bactericidal to the colonies in their weakened state (7). Secretion of pyocyanin is quorum-sensing-regulated, meaning that the bacteria have to reach a certain threshold of growth before this exoproduct is produced and secreted (7). In CF patients, this is one of the major problem-causing exoproducts, causing problems with cellular respiration,

ciliary function, and catalase inactivation (7). In experiments performed in a mouse model testing *P. aeruginosa* virulence, full bacterial virulence was not attained until pyocyanin biosynthesis had begun (7). This exoproduct was also used as an antibacterial agent in this study.

Implications in Healthcare:

In the field of healthcare, *S. aureus* is one of the major problems facing physicians and researchers around the world. Often synonymous with hospital acquired "staph" infections, *S. aureus* can cause many different types of infections in the human body. This bacterium is responsible for infections of orthopedic implants, bacterial septicemia, food poisoning, skin infections, cystic fibrosis superinfections, and many other kinds of infections.

One of the most concerning attributes of *S. aureus* is its ability to adhere to artificial surfaces in the human body and form biofilms (8). A biofilm is essentially a "bacterial city" composed of multiple layers of bacteria that work in unison to secrete compounds that bind them together as a functional unit while simultaneously protecting the colonies against host immune defenses (8). From this "protective city," the bacteria are essentially encased within a protective shell and thus, immune to most if not all outside attack.

Biofilm formation occurs in three phases: attachment, accumulation, and dispersal (8). In the attachment phase, an initial cell, or group of cells, adheres to an artificial surface and begins to replicate. Once a threshold number of cells is reached, the accumulation phase begins and a signal protein is sent out that communicates to the cells in the *S. aureus* colony that they need to secrete a host of proteins to form the biofilm matrix. One of these proteins

is called polysaccharide intracellular adhesion protein, or PIA (8). This protein allows the cells to adhere together and form the biofilm (8). Other secreted proteins contribute to the adhesive abilities of the bacterial colony, as well as the near invulnerability that accompanies the formation of the biofilm (8). In the dispersal stage, cells fragment off of the biofilm and enter the bloodstream, thus causing secondary infections and biofilms to form in other areas of the body (8).

Due to the near impervious nature of biofilms, while inside, *S. aureus* is able to continue to divide and spread via fragmentation without being threatened by any host immune defenses. This means that even the smallest biofilm can result in a large problem if left untreated. Infections such as this can occur on any artificial implantation in the human body, including total joint replacements (TJRs) such as knees, hips, and shoulders, heart valve replacements, and even electrical devices such as pacemakers (8). Because of the impervious nature of biofilms, the only way to treat infections such as these are replacement revision surgeries that involve completely removing the specific artificial surface involved in the infection and replacing it with a new unit. These infections often result in an increased risk of morbidity or mortality due to additional hospital time, additional time in surgery, and the potential for superinfection. This leads to large financial cost and the great potential for decline in patient health (8).

Another potential complication of *S. aureus* is a condition known as bacterial septicemia. Simply described as "blood poisoning," septicemia is a condition characterized by a multisystem, or body-wide, response to a bacterial infection (9). Response can be varied, but to be considered, "severe sepsis," typical symptoms include an elevated white blood cell

count, tachycardia, and in the most severe cases where sepsis worsens to a condition known as "toxic shock," multisystem organ dysfunction and hypotension, or low blood pressure (9). Organ dysfunction typically affects the brain, respiratory system, and cardiovascular system and can result in severe long-term decline in organ function or even death. A few of the most at-risk groups for septicemia are neonates, infants and the elderly. According to a study published in the *Indian Journal of Medical Microbiology*, neonatal septicemia accounts for around a quarter of all neonate deaths in the world and many of the septicemic infections are a result of *S. aureus* (10).

Economically, *S. aureus* also has had a variety of impacts across the world in both the food service and hospital setting. In 2013 alone, antibiotic-resistant infections such as MRSA cost the United States 55 billion dollars in direct and indirect burden on the healthcare industry (11). One example of this is in the case of a total knee arthroscopy revision surgery (TKAR). The average cost of a TKAR is 68,624 dollars (12). This is 16,814 dollars more than the average initial TKA surgery (12). This number reflects the average cost paid by Medicare for each surgery in 2016 and will only continue to increase as the cost of healthcare rises. Expected to rise from 500,000 TKAs per year in 2005 to 3.48 million TKAs per year in the year 2030, if the trend of revisions required for these TKAs continues, the increased burden on the healthcare system will be astronomical (12). Popularity of these surgeries also continues to increase, despite economic cost. Between the years 2009-2010, in the face of a large, countrywide, economic recession, the yearly growth of TKAs increased from 6.1% per year to 13.5% per year (12). A similar trend is being seen in other total joint arthroscopies such as shoulders and hips as well.

Another example of the economic burden created by MRSA infections is related to the increase in length of stay, or LOS, of the patient. A study performed in Switzerland in 2013 demonstrated that MRSA infection results in a hospital stay that is 2.1-4.8 times longer than patients that did not have a MRSA infection (13). Patients incurred an extra cost of 800 Swiss Francs (roughly 850 U.S. dollars) per day (13). This resulted in an additional 6.5 million Swiss Francs (6.9 million U.S. dollars) in healthcare burden being placed on the Swiss economy (13). In the U.S., this problem is even more pronounced. The rate of MRSA infection in the U.S. inpatient setting is 46.3 per 1,000 individuals (14). Due to the difference in population between the U.S. and Switzerland, the economic burden placed on the U.S. economy is even greater than that placed on the Swiss economy. As LOS continues to rise in hospitals and HA-MRSA rates increase, the economic burden will continue to increase and be a drain on economies around the world.

Implications in Athletics and the Community:

S. aureus is also a major concern in the field of athletics, particularly team sports such as football, basketball, volleyball, and weight lifting where players are frequently in contact with one another, increasing the potential for spread of pathogenic strains of bacteria (15). Among these populations, *S. aureus*, or staph, infections are generally thought of as a skin infection characterized by large, pus filled, boils and skin lesions that are highly pathogenic and generally spread quickly throughout a team of athletes if not adequately addressed through environmental sterilization and antibiotic treatment (15). These lesions are often accompanied by cellulitis and pain, and are best treated by draining the boil and ingestion of a broad range antibiotic (15). However, in recent years, the effectiveness of this form of

treatment has seen a drastic decline due to the increased prevalence of strains of *S. aureus* that are resistant to methicillin-based oral antibiotics (15). These strains have come about due to over-prescription by physicians and lack of adherence to the prescribed antibiotic regimen by patients (16). This typically occurs because a portion of the *S. aureus* population survives the initial round of antibiotic treatment and confers this increased resistance to the next generation of *S. aureus* colonies (16). Over multiple generations, entire strains of *S. aureus* have evolved a complete resistance to an antibiotic. In the case of methicillin resistant *S. aureus*, or MRSA, many mutations have evolved to make the bacterial strains resistant to beta-lactam antibiotics such as penicillin and methicillin (12). A few of these mutations lead to a decreased penetration into the cell membrane, the alteration of enzyme active sites, or the degradation of the antibiotic by bacterial enzymes, namely beta-lactamase (16). MRSA infections are concerning to healthcare professionals because many of the antibiotics that were traditionally used to treat these infections are beta-lactams, making most of the common treatments for *S. aureus* infections ineffective. When dealing with MRSA, physicians have traditionally turned to stronger antibiotics, such as vancomycin, to treat these infections. However, in recent years, there has been an increase in *S. aureus* isolates that are even resistant to treatment by vancomycin (17). Dubbed vancomycin resistant *S. aureus*, or VRSA, these new isolates are thought to have emerged due to a combination of the heavy use of vancomycin to treat MRSA infections and the incredible ability of *S. aureus* to adapt quickly to new environmental stressors (17). Already incredibly infectious, certain strains of *S. aureus* also quickly adapt and become nearly impossible to treat, raising many concerns among the medical community.

Infections by *S. aureus*, have also become a problem on college campuses and in schools all over the world. Because *S. aureus* is endemic, or naturally found, on approximately a third of human beings and many of these strains are becoming methicillin resistant, it is not surprising that in a place such as a college campus where thousands of people come together every day, this bacterium would thrive. Typically dubbed community acquired MRSA infections, or CA-MRSA, these infections are distinguished from hospital acquired MRSA infections or HA-MRSA purely by the location in which the patient acquires the bacterium (18). Surprisingly, college campuses have been shown to closely mimic conditions found in hospitals due to the large population of people that are under immune depression due to factors such as stress, antibiotic use, autoimmune diseases, lack of sleep, and many other factors that play into the ability of the immune system to fight infectious diseases. CA-MRSA infections present in a variety of ways ranging from minor soft tissue infections to necrotizing pneumonia, a rare form of pneumonia where the infectious bacterium induces tissue death in the lungs. Although atypical, severe cases are becoming more common as the population of *S. aureus* bacteria continues to evolve increased virulence and infectious capabilities (18).

Implications in Food Service:

Although typically thought of in regard to healthcare and hospitals, *S. aureus* has a reach that extends past healthcare into fields such as food service. For example, a recent study in Brazil assessed the risk that *S. aureus* biofilm formation poses to fish production, one of the major food sources of the country (19). Fish are particularly susceptible to bacterial colonization from *S. aureus* due to the high nutrient content, relatively neutral pH,

and many other favorable environmental conditions of the tissue of the fish (19). This study looked at a few problems facing this part of the Brazilian food supply, and found that one of the main problems was the ability of *S. aureus* to adhere to the stainless-steel surfaces of the machinery used to process the fish and form biofilms, creating the same problem that occurs when biofilms form on artificial medical implants. This resulted in a constant source of contamination that could not be easily destroyed, even when sterilization methods such as heat, chemicals, and pressure were applied (19). *S. aureus* colonization in food supplies is one of the leading causes of food poisoning across the world. This is due to the ability of *S. aureus* to produce a host of toxins, appropriately dubbed staphylococcus enterotoxins, that when secreted from the *S. aureus* cells, induces the common symptoms of food poisoning (20). Roughly 50-70 percent of *S. aureus* strains are able to produce these enterotoxins and these strains account for around 8 percent of food poisoning cases reported in the European Union (20). These enterotoxins, particularly enterotoxin A, induce vomiting, headache, abdominal pain, and diarrhea, and have a particularly rapid onset, often occurring within 1-6 hours of ingestion (20). Infections resulting in food poisoning have occurred frequently in European countries such as France where *S. aureus* is the second most implicated bacteria in food borne diseases, directly following *Salmonella* (20). This shows that *S. aureus* infection is a threat that affects all countries, both developed and undeveloped, without discrimination.

Some of these enterotoxins, such as staphylococcus enterotoxin Q (SEQ), exhibit incredible resistance to heating and pepsin activity (21). This combination of traits means that, if present in food, even cooking cannot eliminate the risk of food poisoning. This toxin also has extreme emetic activity, meaning that it causes severe vomiting, and is not degraded

by peptides in the stomach. Therefore, the effects of this toxin are felt for much longer than many of the other, less stable, enterotoxins (21). Hence, SEQ is an extremely prevalent and present threat to the food service industry. In the case of SEQ, not even the correct method of cooking, cleaning, or food preparation would mitigate the risk of ingesting the active form of this toxin (21). Thankfully, only a few *S. aureus* strains currently have the ability to produce this toxin and therefore this is not an immediate threat to the food service industry.

Recently, *S. aureus* infections have transcended humanity and crossed into livestock as well, resulting in livestock associated MRSA or LA-MRSA (22). This crossover threatens the health of humanity's food supply resulting in many economic implications associated with this food source. This means that *S. aureus* plays an even larger role in economics worldwide than has been historically attributed to the bacterium (22). Directly related to this is the economic loss incurred by food service establishments when *S. aureus* infections occur. In 1984, a study published by Ewen C. Todd about the economic loss resulting from food poisoning outbreaks stated that costs incurred by a food service establishment in the case of a food poisoning outbreak ranged from 16,690 dollars to 1,000,000 dollars which, when the 136 percent inflation rate since 1984 is taken into account, amounts to about 39,375 dollars to 2,359,192 dollars today (23). Cost incurred by a restaurant or business can be attributed mostly to loss of business and potential law suits (23). This steep amount, even on the low end, could bankrupt a small business and shows that, as a population, we need to combat the ability of these bacteria to resist our bactericidal treatments.

S. aureus virulence:

S. aureus is able to be so diverse, adaptable, and destructive due to a variety of weapons, known as virulence factors that this bacterium possesses. These factors include *Staphylococcus* enterotoxin, catalase, peroxidase, beta lactamase, hemolysins, and toxic shock syndrome toxin (11). The focus of this research will be upon a virulence factor, called staphyloxanthin, that provides *S. aureus* with its name.

Staphyloxanthin is a pigment produced by *S. aureus* and is the reason this staphylococcal species is named *aureus*, or gold, in Latin. Like most carotenoid pigments, staphyloxanthin falls in the typical yellow/orange color range under most conditions (23). This pigment has two proposed functions in the *S. aureus* cell, the protection against oxidative stress and the stabilization of the bacterial cell membrane. The first function, protection against oxidative stress, allows the bacteria to persist longer in the host, even while under attack from host immune defenses (23). This is made possible because the pigment acts as an antioxidant, acting to protect the bacterial cell against host defenses such as neutrophils that mainly use free-radical oxidation to attack the *S. aureus* cells (23). This was demonstrated in an experiment that tested the number of *S. aureus* colonies that were able to grow in the presence of increasing concentrations of hydrogen peroxide (H_2O_2), one of the main reactive oxygen species (ROS) that are used by white blood cells in the oxidative attack of pathogens. The results showed that as the concentration of H_2O_2 increased, fewer *S. aureus* colonies were able to grow in comparison to the groups treated with lower amounts of H_2O_2. This led to the conclusion that staphyloxanthin acts as an antioxidizing agent in the bacterial cell (23). In this proposed pathway, staphyloxanthin acts to scavenge free radicals,

protecting the integrity of the bacterial cell from oxidation (23). The pigment presumably acts alongside other compounds such as catalase to neutralize ROS thereby allowing the cell to cope with oxidative stress (23). This is why H_2O_2 was chosen as one of the test reagents in the current study. Clorox bleach was also used as a reagent because the main ingredient, sodium hypochlorite, also acts as an oxidizing agent and has bactericidal properties.

Membrane carotenoids such as staphyloxanthin have also been hypothesized to regulate the *S. aureus* cell membrane stability in much the same way that cholesterol does in the human cell membrane, effectively stabilizing and allowing for adaptability of the membrane based on environmental conditions (24). Alteration of cell membrane fluidity and permeability has been hypothesized to enable *S. aureus* cells to partially detoxify ROSs and allow the cell to better defend against attacks by human white blood cells (24). This characteristic of staphyloxanthin may also help protect the *S. aureus* colonies against attack by antibiotics such as daptomycin that act on the cell membrane level. Daptomycin was chosen as a test reagent for this study due to its method of attack on the bacterial cell membrane. Daptomycin affects seven critical cell membrane proteins that collectively act to regulate the cellular membrane potential in the *S. aureus* cell (25). By altering the membrane potential of the cell, the antibiotic causes cell death because the bacteria lose the ability to transport materials in and out of the cell. This means that they cannot transport ions across the membrane and thus cannot correctly manage its water content, leading to cell lysis or crenation, both of which result in death (25). Daptomycin also causes the conglomeration of bacterial chromosomes, inhibiting the ability of the cell to divide (25).

Purpose:

The two hypothesized functions of staphyloxanthin, protection against oxidation and stabilization of the cell membrane, were tested throughout the course of this experiment using pigment mutants that produce less staphyloxanthin than the wild type strain of *S. aureus* used for experimentation. If staphyloxanthin protects *S. aureus* from host defenses and/or antibiotics because of its antioxidant and cholesterol-like cell membrane stabilization abilities, and we test the antibiotic resistance of mutants that produce differing amounts of staphyloxanthin, then I expect that pigment mutants that produce less staphyloxanthin will be more susceptible to both ROSs and antibiotics that attack at the cell membrane level because the mutants are not able to produce this pigment in the quantity that the wild type is. This will be measured in the form of zone of inhibition data from the Kirby-Bauer antimicrobial susceptibility test. In this test, a plate of Mueller-Hinton agar is inoculated with bacteria and then a filter disk, loaded with an antimicrobial agent, is placed on the plate. After 48 hours of incubation, a clearing around the disk where no bacterial colonies were able to grow is measured and used to determine the susceptibility of the bacteria to that particular antimicrobial agent. This test was also used to compare mutated and unmutated *S. aureus* strains obtained from the American Type Culture Collection (ATCC) to determine if any difference exists between the ROS and antibiotic resistance capabilities between the mutated and non-mutated forms of the *S. aureus*.

MATERIALS AND METHODS

Selection of *S. aureus* Strain:

The strain of *S. aureus* used for this study was chosen based upon the amount of staphyloxanthin pigment that the bacteria produce. ATCC strain 13565 is known for producing large amounts of staphyloxanthin pigment and thus is a model species for pigment study.

Selection of Test Media:

For this experiment, the ideal substrate was one that would allow for rapid growth of the *S. aureus*, allow for easy visibility of pigmentation, meaning the medium was a neutral color, and encourage the production of staphyloxanthin by the *S. aureus* colonies. Seven media were selected to test as potential candidates for use in this experiment. These were Mueller-Hinton agar (MHA) (Difco Laboratories, Detroit, Michigan), tryptic soy agar (TSA) (Neogen Laboratories, Lansing, Michigan), brain-heart infusion agar (BHI) (Neogen Laboratories, Lansing, Michigan), nutrient agar (NA) (Neogen Laboratories, Lansing, Michigan), starch agar, gelatin agar, and Staphylococcus 110 agar (S110) (Neogen Laboratories, Lansing, Michigan). Each medium was streaked in replicates of five with the *S. aureus* strain using a quadrant streak plate method and then incubated at 37 C for 48 hours (26). Growth and pigmentation were ranked on a scale of one to five, one being least amount of growth and least amount of staphyloxanthin produced, and five being the most for both categories. Each plate was scored and the scores of the five replicates of each type of medium were added up to determine the ideal medium for use in further experimentation.

Determination of Mutagenesis Parameters:

The next step of experimentation was the mutation of the original strain of *S. aureus* (ATCC 13565) with the goal of obtaining pigment mutants that produced less staphyloxanthin than the wild-type (WT), or original, strain. This was done using the quadrant streak plate method (26) to inoculate *S. aureus* onto an MHA plate, then immediately exposing the plate to ultraviolet (UV) light at 254 nanometers, the optimal light range for bacterial mutagenesis, with the lid off (27). UV exposure was performed using a model SI-950 UVP incubator as the light source. The first phase of experimentation was determining the optimal distance from the UV light, located at the top of the incubator, and the optimal time of exposure that would result in plates that were densely colonized, but had colonies that were isolated enough to see a distinct pigment difference. It was also important that the colonies were isolated enough to be taken from the plate without risking contamination from other colonies. Initially, six inoculated plates were placed on the bottom shelf of the incubator, located 8 inches away from the UV light source, and exposed for times of 15, 30, 45, 60, 75, and 90 seconds. After exposure in the UV incubator, all six plates were incubated at 37 C for 48 hours. After incubation, plates were examined to determine if any of the treatments met the criteria outlined earlier. Next, the same process was done for the same time periods using the middle shelf, located 5.5 inches away from the UV light source. Finally, the process was repeated using the top shelf of the incubator, located 3 inches away from the UV light source. A set of six plates was inoculated using the quadrant streak plate method and exposed on the top shelf of the UV incubator for 2, 4, 6, 8, 10, and 12 seconds (26).

Mutagenesis and Mutant Phenotypic Isolation:

The next step was to isolate colonies of *S. aureus* that exhibited decreased pigmentation after exposure to UV light. Eight plates, labeled A-H, were streaked using a quadrant streak plate method and exposed for seven seconds on the top shelf of the UV incubator. The plates were then incubated for 48 hours at 37 C. After incubation, the plates were examined and colonies that exhibited decreased pigmentation were selected and streaked onto fresh MHA plates that had been divided into four sections to optimize use of media. Eighteen possible pigment mutants were obtained (3 from plate A, 4 from plate B, 3 from plate C, 3 from plate D, 4 from plate E, and 1 from plate G). Each potential pigment mutant was given its own section of an MHA plate. These plates were then incubated at 37 C for 48 hours to determine if the colonies were pigment mutants or just *S. aureus* colonies that had turned off their pigment temporarily, a trait that *S. aureus* is known for when it is not under environmental stress. The six mutants that retained their lack of pigmentation were then streaked onto separate MHA plates and incubated as above. This transfer method was performed a total of three times to ensure the new mutants retained their diminished pigmentation.

Confirmation of Phenotypic Mutations:

To determine that the newly isolated colonies were not contaminants (other bacterial species or other *S. aureus* strains), all presumed mutants underwent latex agglutination testing (Hardy Diagnostics, Santa Maria, California). Testing was performed using a sample of the wild type colony, designated WT, and each of the mutated bacterial strains (D1, D3, and E1) isolated in the previous experimental step. A Staph API test (bioMèrieux

Laboratories, St. Louis, Missouri) was also performed on the mutants and the WT by inoculating a series of 20 microtubes filled with various reactants used for the identification of different species of *Staphylococcus* and including different strains within these species. The entire panel was incubated for 24 hours at 37 C. After incubation, each test was assigned a numerical value based on whether the test was positive or negative and this number was used to identify the species of bacteria based on the manufacturer's data base. If the experimental group of colonies matched the numerical code of the WT sample, it was then assumed that it was the same strain of *S. aureus*.

Kirby-Bauer Antibiotic Susceptibility Testing:

Reagents used in this portion of experimentation were 3% hydrogen peroxide, Clorox brand bleach, pyocyanin (Sigma Laboratories, Mumbai, India), daptomycin (Sigma Laboratories, Mumbai, India) HQNO (Enzo Laboratories, Farmingdale, New York), and blank discs (Hardy Laboratories, Santa Maria, California).

The purpose of this phase of experimentation was to test if differential antibiotic resistance exists between the 4 samples using the Kirby-Bauer method to test antibiotic susceptibility of each strain. The method utilizes paper discs that were impregnated with the test chemical. Also known as the single-disk diffusion method, the Kirby-Bauer method involved inoculating a 0.85% saline solution with a bacterial isolate until it matched with a turbidity standard (McFarland 0.5 turbidity standard) (Sigma Laboratories, Mumbai, India) (28). After the saline suspension was created, a cotton swab was dipped into the saline solution for 3 seconds and then rolled along the inside of the test tube to eliminate excess moisture. The swab was then streaked across a standardized MHA plate containing 25 mL of

agar. The plate was then rotated 90 degrees and streaked again and rotated & streaked once more to ensure that the entire plate was coated in bacteria as an even inoculum. After inoculation, blank Hardy discs were placed equidistant apart on the MHA plate (28). For each trial per strain, 2 plates were used. One plate contained the discs inoculated with full strength 3% H_2O_2 (H) and Clorox bleach (C). The other plate contained the HQNO (HQ), Pyocyanin (P), and Daptomycin (D) discs. Once the discs were placed, the discs were loaded with the various antibacterial agents using a micropipette. Four microliters of antibacterial were loaded onto the H, C, and P discs. 2 microliters of HQ were loaded onto a disc and 20 microliters of D were loaded onto a disc. Once all discs were loaded, the MHA plate was incubated at 37 C for 48 hours (1). This constituted one data point for one strain. This was repeated 30 times for each strain.

After incubation, the diameter of each zone of inhibition was measured in mm at the widest part of the zone. Areas where enhanced pigmentation in the borders of the zones of inhibition were obviously exhibited were also noted. The results were then tabulated in Microsoft Excel for further analysis.

Statistical Analysis:

All statistical analysis was performed using R version 3.3.1. Data from Microsoft Excel was imported into R for analysis. Means and standard error for each treatment per strain were calculated and used to make a scatterplot with standard error bars. Quantile-quantile plots were then performed on each strain for each treatment to assess the normality of the data. A Fligner-Killeen test was then run on the data to determine homoscedasticity of the data set. Next, the Akaike's An Information Criterion, or AIC, was used to determine

which model best represents the data. After choosing the simplest model, a one-way analysis of means (ANOMA) was performed to determine if a significant amount of difference existed between the means of the groups. If a statistically significant difference ($p<0.05$) was detected, then Welch's two-way t-test was used as a post-hoc test to correct for homoscedasticity and to determine the relationships between strains and treatments given the data.

RESULTS

Selection of Media:

Mueller-Hinton agar was chosen as the medium for the remainder of the experiment. Not only did bacteria inoculated on this medium show the most volume of growth and amount of pigmentation produced, it is also the standard medium used for Kirby-Bauer antibiotic susceptibility testing (28) (Table 1).

TABLE 1. Ranking of various media based upon volume of growth and amount of pigment produced by the bacterium, S. *aureus*

Media	Volume of Growth (1-5)	Amount of Pigment Produced (1-5)
Brain-Heart Infusion Agar	4	4
Gelatin Agar	0	0
Mueller-Hinton Agar	5	5
Nutrient Agar	2	2
Staphylococcus 110 Agar	4	5
Starch Agar	3	2
Tryptic Soy Agar	4	3

Determination of Mutagenesis Parameters:

In the initial trial, six plates were inoculated, incubated on the bottom shelf of the incubator, and exposed to 254 nm UV light for 15, 30, 45, 60, 75, and 90 seconds. All of these plates exhibited growth, but the growth was far too dense to isolate individual mutated colonies. Next, the same process was done for the same time parameters on the middle shelf of the incubator and although less dense, the growth was still slightly too dense and was not exhibiting any difference in pigmentation that signaled mutagenesis in the bacterial cell. Finally, the same process was repeated on the top shelf of the incubator for 2, 4, 6, 8, 10, and 12 seconds. The results showed that UV exposure for less than 6 seconds did not show sufficient bactericidal properties and resulted in growth too dense to isolate individual colonies that exhibited mutagenesis. Conversely, above the 8 second exposure mark, the UV was far too bactericidal and there were not enough colonies on the plate to acquire mutants with any significant frequency. However, between 6 and 8 seconds of exposure, colonies were sufficiently dense on the plate, able to be easily isolated, and showed differentiation in the amount of pigment produced by isolated colonies, indicating possible mutagenesis (Fig. 1).

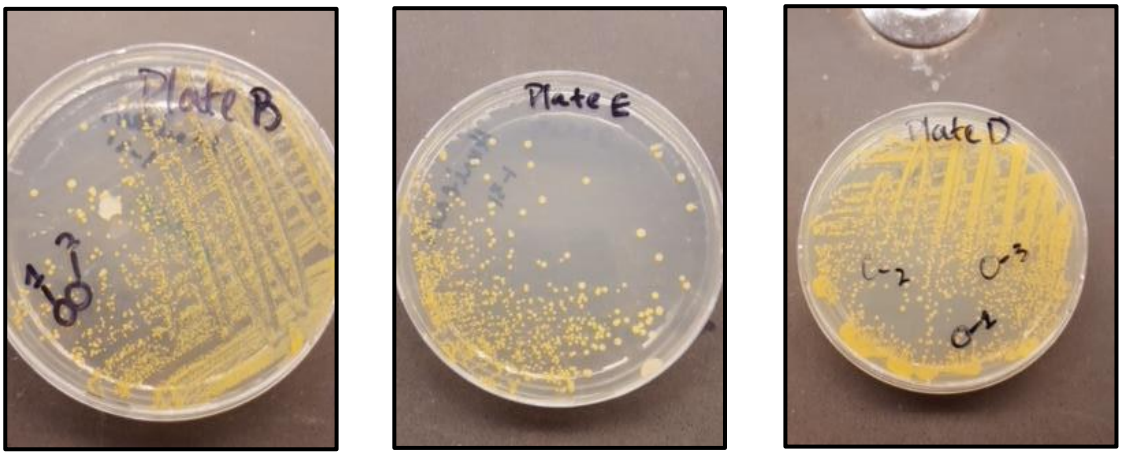

FIG. 1. MHA Plates exposed to 254 nm UV light on the top shelf (3 inches away from light source) of the Model SI-950 UVP incubator for 7 seconds

Mutagenesis and Mutant Phenotype Isolation:

Eighteen possible phenotype mutants were isolated from the eight plates that were exposed to UV light for seven seconds on the top shelf of the UVP SI-950 UV incubator. Of these eighteen potential mutants, only six mutants retained their differential pigmentation through the initial transfer and incubation cycle. After the following three transfers performed on the remaining six mutants, only three of the six maintained their differential pigmentation. These were the E1, D3, and D1 mutant strains. The D3 strain exhibited a complete lack of staphyloxanthin production through these transfers and the E1 and D1 cultures exhibited a reduced production of staphyloxanthin in comparison to the WT strain (Fig. 2).

FIG. 2. Four mutants that retained differential pigmentation after 3 transfers streaked on MHA. The WT strain, lower right quadrant, is the most heavily pigmented, E1 and D1,lower left and upper left quadrant, respectively, show decreased pigmentation, and D3, upper right quadrant, is completely non-pigmented.

Confirmation of Phenotypic Mutations:

All four experimental groups tested positive in the latex agglutination test (Fig. 3). This indicates that all four are most likely *S. aureus*.

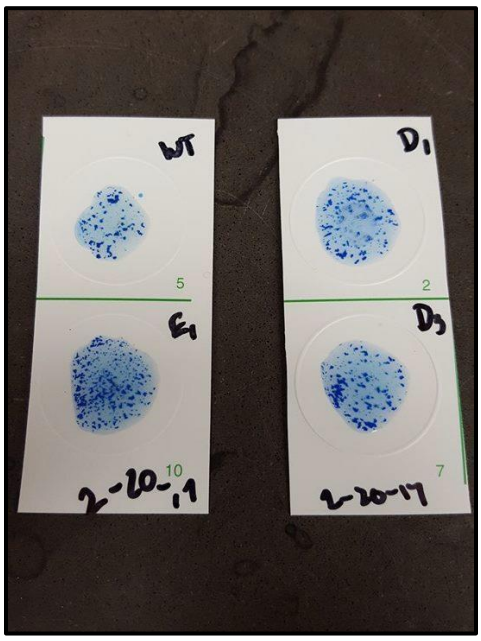

FIG. 3. Latex agglutination test performed on each of the experimental groups. *S. aureus* is able to cause latex to clump, and thus gives the characteristic appearance seen above in all four tests

From the API 20S panel, all four groups yielded the profile number 6736150, which according to the manufacturer's data base profile key, matches with *S. aureus* (Fig. 4). Because all of the groups yielded the same profile number, it can also be presumed that all four are the same strain of *S. aureus*. Due to these results, it is feasible and likely that the three experimental groups are mutants of the same WT strain and not contaminants from the environment.

FIG. 4. API 20S test results of the three mutants and the WT strain of the bacterium. The WT was used as the positive control in this experiment as it was the original strain in the experiment.

Kirby-Bauer Antibiotic Susceptibility Testing

The Kirby-Bauer Antibiotic Susceptibility testing method was used to determine whether the different mutants of *S. aureus* exhibited differential resistance to the antibiotics used (Fig. 5). One difference that was noted between the pigmented and non-pigmented strains was the increase in pigment production around the edge of the zones of inhibition in the H treatment (Fig. 5). This was only seen in the pigmented strains, E1 and WT, and not in the non-pigmented strains, D1 and D3 (Fig. 5).

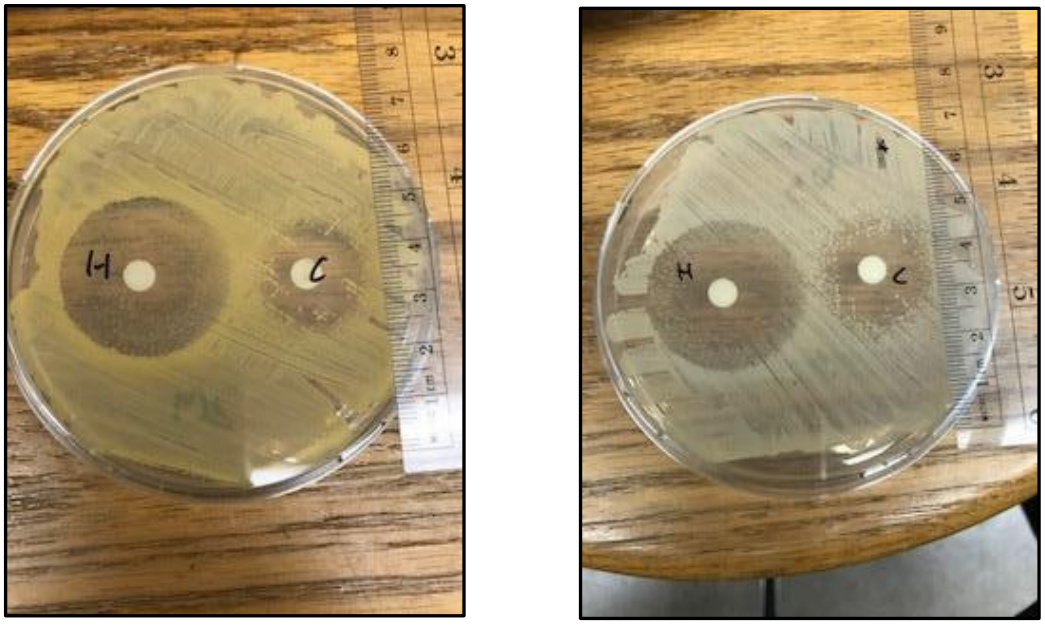

FIG. 5. Left: Kirby-Bauer Susceptibility Test run on Mueller-Hinton Agar. Zones of inhibition are measured at the widest point of the zone of clearing in mm. In the plate on the left, increased pigmentation can also be noted around the edge of the zone.

Thirty iterations of the Kirby-Bauer method were performed in most treatments, the only exception being the D treatment where only 13 iterations were performed due to lack of antibiotic (Table 2). Means were calculated from the data gathered from the Kirby-Bauer testing and analyzed using a variety of statistical methods described below (Table 2).

TABLE 2. Mean zone of inhibition size and number of measurements of Kirby-Bauer testing. Groups are divided by treatment and strain

Treatment	Strain	# Measurements	Mean Zone Diameter
C	WT	30	21.2
D	WT	13	15.7692308
H	WT	30	30.7666667
HQ	WT	30	0
P	WT	30	25.2666667
C	D3	30	22.5666667
D	D3	13	22.5384615
H	D3	30	30.0333333
HQ	D3	30	23.2666667
P	D3	30	32
C	E1	30	22.0333333
D	E1	13	19
H	E1	30	32.2333333
HQ	E1	30	0.7333333
P	E1	30	25.4666667
C	D1	30	21.8333333
D	D1	13	21.2307692
H	D1	30	26.2666667
HQ	D1	31	11.2903226
P	D1	30	29.8333333

*Treatment abbreviations are as follows: C=Clorox, H=Hydrogen peroxide, D=Daptomycin, HQ=HQNO, and P=Pyocyanin

The Fligner-Killeen test for homoscedasticity of the data indicated that the data were not homoscedastic (χ^2=67.933 df=17 p<0.001). Quantile-quantile plots determined that the

data were mostly normal with a few small violations to the normality. The AIC function determined that the most simple and appropriate model to assess the data was to analyze measurements given strain or treatment in the form of a one-way analysis of means (ANOMA). ANOMA analysis determined that no difference existed in the mean measurements of the zones of inhibition (ZOI) in the H (p=0.05972) and C (p=0.05037) treatments (Fig. 6). Differences were found to exist in the D (p=3.096e-07), P (p=9.379e-16), and HQ (p=8.749e-06) treatments (Fig. 6). In the groups where differences were exhibited, Welch's two-way t-tests with a Holm adjustment were used as a post hoc test to determine where the differences existed within the treatment. Among the D treatment, the WT group varied from the E1 (p_{adj}=0.00886800), D1 (p_{adj}=0.00010808), and D3 (p_{adj}=0.00001236) groups (Fig. 6). The E1 group varied from both the D1 (p_{adj}=0.00609000) and D3 (p_{adj}=0.00004991) groups and the D1 group varied from the D3 (p_{adj}=0.02916000) group (Fig. 6). In the HQ treatment, no inhibition was shown by the treatment in the E1 and WT groups, therefore for analysis purposes, these sets were excluded from the data analysis for this portion. Both the D1 and D3 zones were inhibited by the HQ treatment, indicating that a difference existed between these two groups and the two groups that were not inhibited (Fig. 6). Statistical difference was also seen in the means between the D1 and D3 (p_{adj}<0.0001) groups (Fig. 6). In the P treatment, the WT group mean did not vary from the E1 (p_{adj}=.079680) but did differ from both the D1 and D3(p_{adj}=0.18062) groups (Fig. 6).

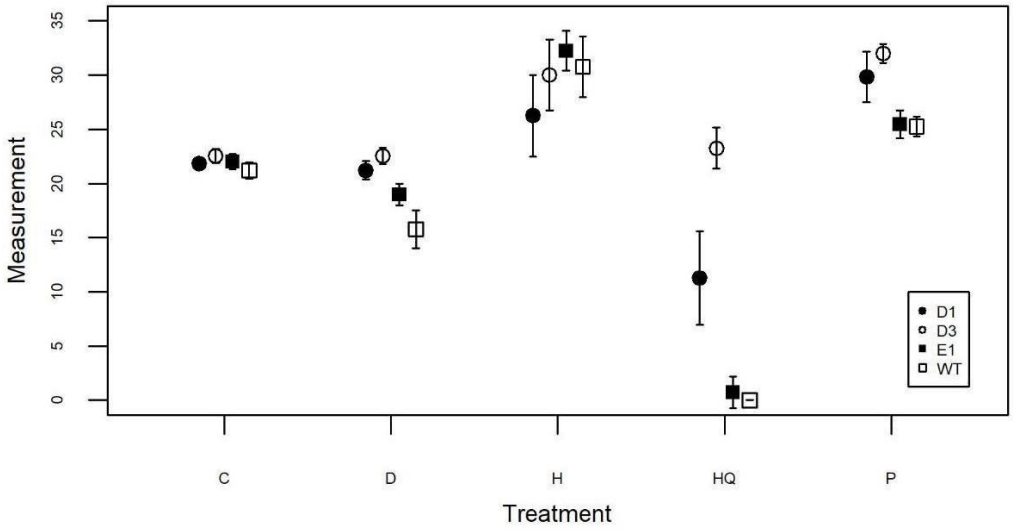

FIG. 6. Scatterplot depicting the means of each strain separated by which antibacterial agent they were treated with. Measurement on the Y-axis is in mm. Differences can be clearly seen in the HQ and P treatments between the E1 and WT groups and the D1 and D3 groups. Slight differences in the D and H treatments may also be noted

*Treatment abbreviations are as follows: C=Clorox, H=Hydrogen peroxide, D=Daptomycin, HQ=HQNO, and P=Pyocyanin

DISCUSSION

S. aureus was shown to grow best on the Mueller-Hinton agar (MHA). The bacteria showed denser growth and a higher rate of pigment production on this agar medium compared to others tested. MHA is a general-purpose medium that is used for growing many different types of bacteria. It is also the standard agar used for the Kirby-Bauer antimicrobial susceptibility testing method (29). Because it yielded better results and could be continuously used throughout experimentation, it was chosen as the test medium for this study.

Ultraviolet (UV) light has been shown as a consistent progenitor of mutagenesis pathways in bacteria (30). This has been demonstrated in a wide variety of bacteria spanning multiple taxa (30). One of the initial bacterial species where this method of mutagenesis was performed was *E. coli* (30). In *E. coli*, mutagenesis can be attributed to two distinct effects of the radiation, the blocking of DNA synthesis, which initiates both RNA and protein synthesis systems in order to repair the functionality of the DNA synthesis system, and photochemical modifications that result in mutation as subsequent DNA begins to repair (30). This results in DNA that is "semiconservative" or mostly the same as it was prior to exposure, but also carries distinct and quantifiable differences to the unexposed DNA (30). One hypothesis for explaining the bacterial response to UV light is known as the "SOS" hypothesis (31). This hypothesis proposes that UV mutagenesis is dependent upon the product of a multitude of genes, one of which is the *recA*$^{+}$gene (31). This gene is only activated in times of UV exposure when the *E. coli* cell is experiencing extreme stress (31). When active, the *recA*$^{+}$ gene is responsible for postreplication repair of *E. coli* DNA strands and is known for being error prone, thereby causing mutations in the genome of the bacteria (31). Studies have

shown that *S. aureus* is similarly affected by UV exposure (32). *S. aureus* has been shown to be equally susceptible to both the bactericidal and photobiological effects of UV light, even exhibiting many of the same mutagenesis pathways seen in *E. coli* (32). It has also been demonstrated that quantifiable physiological mutations can be induced in *S. aureus* cells by exposure to UV light (33). One example of this was in an experiment performed by Swarupa et. al. In this experiment, the overexpression of the product of the gene streptomycin 3''-adenylyltransferase 1, was induced by exposure to UV light (33). This led us to question whether or not differential pigment production could be induced in the same fashion. To accomplish this, exposure to UV light at 254 nm for 6-8 seconds at a distance of 3 inches from the light source was determined to be the optimum distance and time of exposure for mutagenesis while still supporting enough bacterial colonies on the plate to increase the chance that mutants would be seen in high enough frequency to be detected and isolated. After exposure to the UV light and subsequent incubation and growth, eighteen possible mutants were isolated from eight plates. They were each taken and restreaked onto another MHA plate and incubated. This process was repeated 3 times because *S. aureus* has the ability to increase and decrease its pigmentation in response to environmental factors. This was demonstrated in our experimentation around the edge of the zones of inhibition in the H_2O_2 treatment. Restreaks were necessary to ensure that what we were seeing weremutants that were not able to produce as much staphyloxanthin pigment rather than non-mutated bacteria that are producing less staphyloxanthin. After 3 rounds of re-culturing, only 3 potential pigment mutants remained that had maintained their differential pigment production.

The next step in the determination of mutagenesis was to ensure that these potential pigment mutants were not just contaminants on the plate. This was performed in two steps. The first was to confirm that the isolates were *S. aureus* and the second was to ensure that if we did have *S. aureus*, they were all the same strain of *S. aureus* that differed only in the amount of pigment produced. The first determination was done using a latex agglutination test. The latex agglutination test is one of the standard methods for identifying *S. aureus* in the laboratory setting (34,35). These tests are commonly used to quickly identify *S. aureus* by binding to surface antigens and cellular exoproducts unique to the cell of *S. aureus* and not other *Staphylococcus* species (34,36). A few of these unique properties include the enzyme, coagulase, which is one of the main factors that causes the clumping of latex in this test, and the presence of a cell wall containing protein A (36). This test has been shown to have a 96-98% sensitivity and specificity to the *S. aureus*, meaning the prevalence of both false negatives and false positives resides in the 2-3% range, making it a useful assay for the discrimination between *S. aureus* and other bacterial species (35). In this test, particles in the solutions used precipitate with antigens located on the surface of the bacteria or exoproducts secreted by the bacteria (35). This causes a clumping to be seen almost immediately and in the case of our potential mutants, all tested positive and thus were determined to be *S. aureus*.

The second determination, that all the *S. aureus* were the same strain, was necessary because *S. aureus* is endemic to the human body in 30-40% of the population and could therefore have been a contaminant from a human host (37). This was done using an API 20 staph panel. In this panel, the mutants were tested against 20 different compounds designed

to test properties of different staphylococcal species for the purpose of differentiating between and identifying species and strains within the species *Staphylococcus* (38). This process is known as biotyping and has been shown to be extremely effective in identifying species of staphylococci (38). When using this test to identify bacterial species, an index is used in which multiple different species numbers are assigned to one species because many biological variants within each species is common, for this particular API panel, there are several hundred possible biotypes for *S. aureus*. This is why for the purposes of this experiment, this test was used to determine identity between strains. The likelihood that a contaminant wild-type strain of *S. aureus* from a human host or the environment exactly matches the experimental strain of *S. aureus* is extremely low, leading to the assumption that we were still dealing with mutants of the experimental strain rather than environmental strains that were non-pigmented. Based upon these results from the latex agglutination and API tests, we assumed that for the purposes of this study, the only difference between the groups was the differential production of the pigment, staphyloxanthin. It should be noted that there was no DNA analysis run on these groups to determine the exact mutations that had occurred as a result of the exposure to UV light. Ideally, whole genome sequencing (WGS) would be run on these mutants to determine if any other mutations are present that may be causing a differential response to anti-microbial treatment (39). However, that exceeded the scope and budget of this experiment. Although WGS has significantly dropped in price and increased in specificity in recent years, it still costs around 75-150 dollars per identification to obtain raw data, not even including the cost required for analyzing and representing these data (39). It is unlikely that the only mutations that occurred were in the

pigment production pathway. However, due to the similarity in the colony morphology and the fact that they all originated from the same strain and exhibited the same API results, the study continued under the assumption that the only important variable that would be taken into account was the differential pigment production.

Staphyloxanthin carotogenesis is controlled, in part by the genes *crtM* and *crtN*, which encode two of the major aspects of the staphyloxanthin pathway, dehydrosqualene synthase and dehydrosynthase desaturase, respectively (40). It has been shown that eliminating the function of the *crtM* gene completely inhibits the ability of the *S. aureus* colony to produce staphyloxanthin (40). These genetic knockouts were shown to be much more susceptible to killing by hydrogen peroxide than the fully pigmented colonies with the intact *crtM* gene (40). This experiment led our laboratory to question two things, one whether we could generate pigment mutants using only UV light and two, whether or not this differential response would be seen in a similar fashion in treatments by other antimicrobial agents that attack aspects of the bacteria that staphyloxanthin directly influences. As shown by Liu et. al. in 2005, staphyloxanthin acts to help protect the *S. aureus* cell from oxidation by ROS such as hydrogen peroxide. However, it also acts to stabilize the cellular membrane by regulating the fluidity of the cellular membrane (25).

In the Kirby-Bauer susceptibility testing performed in the present study, one of the interesting things that was noted was the increase in pigment production around the edge of the zones of inhibition by the E1 and WT groups in the H_2O_2 treatment. This is a phenomenon that has been documented a few times in our laboratory when staphyloxanthin-producing *S. aureus* is placed in close proximity to *P. aeruginosa*. This makes sense as *P.*

aeruginosa has been documented to increase or even induce pigmentation in *S. aureus* colonies, and this leads us to believe that *S. aureus* may be using a similar pathway to protect against oxidation in the H_2O_2 treatment. However, the phenomenon had not been previously seen in interactions between H_2O_2 and *S. aureus*. This may point to the abilityof staphyloxanthin to act as an antioxidizing agent that lends some extra protection to the bacterial colony when under oxidative stress. This effect of staphyloxanthin has been noted by prior research sources and has been proposed as one of the major functions of the pigment (23). Staphyloxanthin has been shown to scavenge free radicals and allow the bacterial colonies that produce it to have a marginally higher degree of oxidative protection than colonies that are not able to produce the pigment (9). In this experiment, however, no statistical difference was seen between the zones of inhibition of the different strains for the H_2O_2 or Clorox treatments. The combination of the increased pigmentation around the zones and the mean zone sizes may support the idea that although the staphyloxanthin acts as an antioxidant, it is not strong enough to make a statistical amount of difference. The staphyloxanthin in the Clorox and the H_2O_2 treatments, did not affect the resistance exhibited by any of the groups. It was noted in prior research that staphyloxanthin most likely did not play as large of a role in protection against oxidation as other factors such as catalase and superoxide dismutase (SOD), however how small of a role it plays was not discussed (23).

Also seen in the Kirby-Bauer testing were differences between the groups in response to Daptomycin treatment. Daptomycin is a lipopeptide antibacterial that exhibits rapid activity against a wide range of gram-positive cocci (41). The mechanism of action of this antibacterial is to bind to the cell membrane and affect seven key transmembrane proteins

that regulate the osmotic potential of the bacterial cell membrane (41). This causes a disruption in DNA, RNA, and protein synthesis and quickly thereafter, cellular death (41). Daptomycin is an important antimicrobial that is used in treating both methicillin and vancomycin resistant *S. aureus* infections and has been shown to be extremely effective when typical beta-lactam antibiotics prove to be ineffective (41,42). To date, no mechanisms of resistance to daptomycin have been demonstrated by *S. aureus*, leading to the increased use of this antibacterial in high doses to treat difficult *S. aureus* infections (43). Results obtained from this study showed that the group that was able to produce the most pigment, the non-mutated WT group, exhibited the most resistance to the treatment, followed by the other pigmented group, E1, and then the two non-pigmented groups, D1 and D3. This matches the hypothesis that the presence of staphyloxanthin in the cellular membrane of *S. aureus* protects it, to an extent that is measurable, from attack by cell membrane active antimicrobials such as daptomycin. This could become particularly important in combination treatments that involve both anti-virulence and anti-microbial compounds. Anti-virulence therapies are defined as treatments that seek to alter the bacteria physiologically without killing it in a way that makes the bacteria more susceptible to host immune defense or traditional antimicrobial therapy (44).

Another result seen in the Kirby-Bauer testing was in the *P. aeruginosa* exoproduct treatments. In the pigmented groups, E1 and WT, there was absolutely no susceptibility to the HQNO (HQ) treatment. However, in the non-pigmented groups, D1 and D3, clear zones of inhibition could be seen, some as large as 25 mm in the D3 group. This is particularly interesting because HQNO acts on the electron transport chain, particularly on the enzyme

nitrate reductase (5,45). Nitrate reductase facilitates the electron transfer from lactate to nitrate, an intermediate step in the bacterial electron transport chain (5,45). HQNO has been shown to have a strongly inhibitory effect on transfer of electrons (5,45). This may be related in some way to staphyloxanthin because nitrate reductase, like staphyloxanthin, is found in the bacterial cellular membrane, indicating that staphyloxanthin may be exhibiting a form of shielding or protective effect for the nitrate reductase, partially protecting it from HQNO attack, in the pigmented colonies (5,45). Staphyloxanthin had not been previously hypothesized to have any part in preserving the integrity of the electron transport chain; however, this association makes some sense as the electron transport chain is located in the cellular membrane of bacteria, not specific organelles like in eukaryotic organisms (5,45). The stark difference between the pigmented and non-pigmented groups, however, indicate that staphyloxanthin may have more of a purpose in protecting components important in electron transport and respiration rather than in protecting against oxidation. In the pyocyanin treatment, the same phenomenon was seen, just to a different degree. Pyocyanin also acts on the electron transport and respiration mechanisms of *S. aureus,* however the mechanism of action is different. Pyocyanin is a pigmented quorum-sensing-regulated exoproduct of *P. aeruginosa*. This exoproduct is one of the main virulence factors of *P. aeruginosa* and has been shown to have cytotoxic effects on both human epithelial cells and bacterial cells alike (7). Pyocyanin has been shown to be toxic to both respiring and non-respiring *S. aureus* cells (7). In non-respiring cells, pyocyanin acts as a ROS, oxidizing the bacterial cell (7). However, in respiring cells, this pigment acts to trigger the shift to a small colony variant (SCV) phenotype (7). These SCV colonies are less pigmented than their normal counterparts

and exhibit a non-functional electron transport mechanism (45). Although less efficient, this SCV phenotype has been demonstrated to be more hardy than the normal variant and is one of the main reasons that *S. aureus* is able to survive so long in the presence of *P. aeruginosa* in chronic infections such as cystic fibrosis (45). This phenotypic variant has been isolated in roughly 24% of patients with chronic cystic fibrosis infections and is significantly more resistant to antibacterial attack than the non-SCV phenotype (7). The mean zone of inhibition size for the pigmented groups was around 25 mm and for the non-pigmented groups was nearly 30 mm and 32 mm in the D1 and D3 zones, respectively, indicating that the staphyloxanthin pigment in the membrane had some sort of shielding effect against the mechanism of action unique to pyocyanin.

The results seen in the treatments using the *P. aeruginosa* exoproducts begin to make sense when the frequency with which these two bacteria coinhabit the same space is taken into account. *P. aeruginosa* and *S. aureus* coinhabit a variety of infections such as cystic fibrosis, burn wounds, diabetic ulcers, and deep cuts. It is logical that as these two bacterial species have inhabited the same niches, they have also each evolved a variety of ways to win the ecological battle when they do come into contact. This also explains why coinfections involving these two bacteria, especially in the case of cystic fibrosis, can be continuous for 10-20 years. Each of these bacteria are able to suppress and keep in check the growth of the other. In the case of *P. aeruginosa*, it secretes a whole host of compounds that are specifically targeted towards *S. aureus* (4). These compounds have no other purpose to the *P. aeruginosa* cell than winning the battle against *S. aureus* and often lend it the competitive edge against *S. aureus*, especially in chronic infections, such as cystic fibrosis. In cystic

fibrosis, the initial bacterial infection is often caused by *S. aureus*, however over the duration of the infection, *P. aeruginosa* usually coinhabits and is the pathogen that ends up killing the patient. In *S. aureus*, it has also been shown that in certain circumstances, *P. aeruginosa* induces the overexpression of staphyloxanthin pigment by *S. aureus* (44). This is made possible by the secretion of compounds by *P. aeruginosa* that induce upregulation of the genetic pathways that coordinate with carotogenesis (44). Also a result of these same molecules is the upregulation of the production of the enzyme, catalase, that helps protect *S. aureus* cell from oxidation by ROS (44). Interestingly enough, when *P. aeruginosa* is present in small quantities, as is common in the early stages of many of these coinfection circumstances, *P. aeruginosa* induces a higher degree of pathogenicity in the *S. aureus* cell, helping the bacteria to survive longer rather than being suppressed as is seen later in the course of the infection (44). As the infection progresses, *P. aeruginosa* becomes more prevalent and the concentration of exoproducts in the infection space increases, resulting in the inhibition of *S. aureus.* Competition between these bacteria, and others in the case of polymicrobial infections, often results in the increased pathogenicity of multiple bacteria and thus the increase in the severity of the infection (44). Polymicrobial infections are becoming extremely prevalent as these bacteria become more resistant to antimicrobials and continue to induce increased virulence in each other (44).

Moving forward, various conclusions can be drawn from this study. One of the major conclusions is the need for increased research into anti-virulence therapies. One proposed method of this phenomenon is through the knockout of the gene Cold shock protein A (CspA) (45). CspA is a cold shock protein found in *S. aureus* that is tied to the Sig-B

mechanism that is required for maximum pigment production (45). In cells where this protein has been genetically knocked out, the bacteria are unable to produce any staphyloxanthin pigment (45). In this example, the lack of the ability to produce staphyloxanthin would result in an increased susceptibility to certain antimicrobials such as daptomycin. Another major advancement in this field was the discovery of the antibiofilm, antipathogenic, and anticarotenogenic properties of the chemical L-ascorbyl 2,6-dipalmitate (ADP). This is important to a variety of fields for multiple reasons, one of the major ones being the antibiofilm properties. Biofilm formation by *S. aureus* causes many problems in both the medical and food service industries (16). By inhibiting the ability of *S. aureus* to form biofilms, billions of dollars could be saved just in the reduction of revision surgeries alone (16). In the food service industry, a treatment such as this could be equally as helpful. In the example used earlier, Brazil's fish production industry, where *S. aureus* biofilms that could not be destroyed were constantly forming and causing problems by contaminating the food source, a treatment that inhibits the bacteria's ability to adhere to a surface would result in less money being spent on cleaning equipment and purchasing new machines to combat the biofilm formation (15). This would be beneficial to not only the producer, but also to the consumer, resulting in less food-borne illnesses and potentially eliminating one of the major causes of food poisoning worldwide. These therapies are promising in the face of the increased resistance to antimicrobials that bacteria such as *S. aureus* have been exhibiting in recent years. Because these new anti-virulence treatments do not affect the ability of bacteria to grow and reproduce, they also do not induce rapid evolutionary responses toward increased pathogenicity as is seen currently with antimicrobial treatments (46). Anti-

virulence treatments have the added benefit of not harming the endogenous flora already present on the human body (47). This means the chance that opportunistic pathogens will be able to cause infections by filling the ecological gap created by the loss of normal flora is much less likely than with traditional antimicrobial treatment (47). This would result in bacterial virulence being naturally inhibited by other bacteria and a sort of "crowd control" phenomena being seen, keeping pathogenic bacteria in check more naturally and through better means than bacteriocide (47).

Anti-virulence treatment with ADP also has the potential to be extremely important due to its anticarotenogenic property. By definition, an anticarotenogenic compound would suppress *S. aureus* ability to produce the pigment staphyloxanthin, which has been shown to be a significant factor in *S. aureus* ability to resist antimicrobial attack. This was demonstrated in the *P. aeruginosa* exoproduct (HQNO and Pyocyanin) and Daptomycin treatments of the Kirby-Bauer susceptibility testing. By knocking out the staphyloxanthin production, we make the bacteria much more susceptible to routine antimicrobial treatment and host clearing (47). This phenomenon has also been demonstrated from other sources. In an experiment by Song et al., one of the introductory steps in staphyloxanthin biosynthesis was inhibited by using a product that is produced by a wide variety of microorganisms (48). The introduction of this compound to the *S. aureus* cell resulted in bacterial colonies that were unable to produce pigmentation and thus were rendered more susceptible to other treatments, such as hydrogen peroxide (48). Experiments such as this and many others show the potential promise that lies in the use of anti-virulence treatments as a way to combat not only *S. aureus* infections, but also the antimicrobial resistance epidemic as a whole.

The results of this study supported my hypothesis that the removal of staphyloxanthin from the bacterial cell resulted in a higher degree of antimicrobial susceptibility in most cases. The data indicated three major points about staphyloxanthin. The first point is that staphyloxanthin did not function to protect the bacteria from oxidation via H_2O_2 or Clorox to a statistically significant degree. This was indicated by the Kirby-Bauer antimicrobial susceptibility testing. The presence of H_2O_2 however, did increase the pigment production seen around the edge of the zones of inhibition in the pigmented groups, indicating that staphyloxanthin does probably play a minute role in antioxidation. The second point is that staphyloxanthin does act to protect the bacterial cell from antimicrobials that affect the cellular membrane. This confirms that staphyloxanthin is important in the protection of the bacterial cell against cellular membrane attack via antimicrobials altering the fluidity of the bacterial cellular membrane and that this quality of the pigment is protective in function. The final point is that staphyloxanthin protects against electron transport altering treatments, suggesting a role in maintaining the integrity of the electron transport chain in *S. aureus*. This role of staphyloxanthin had not been previously demonstrated and requires more research to determine by what exact method this occurs in the *S. aureus* cell.

S. aureus is one of the most concerning pathogens facing our world today. It is a problem not only to the field of medicine, but also to the fields of food service, community health, sports, and even economics. This bacterium has a variety of factors that serve to increase its virulence, such as the pigment staphyloxanthin, allowing the bacterium to be dangerous in a variety of ways that are unique to this pathogen. Staphyloxanthin serves a variety of roles in the *S. aureus* cell that have been shown to include an extremely minute

amount of protection against oxidation and also significant protection of the bacterial cell membrane and electron transport chain. These functions have been shown to significantly increase the amount of resistance that the bacterium displays against a variety of antimicrobial agents and demonstrates that continued research into therapies that are able to reduce the virulence of this bacterium is necessary.

REFERENCES

1. **Hannsen, A.M., Kindlund, B., Stenklev, N.C., Furberg, A.S., Fismen, S., Olsen, R.S., Johannessen, M., Sollid, J.U.E.** 2017. Localization of *Staphylococcus aureus* in tissue from the nasal vestibule in healthy carriers. BMC Microbiol. **17:**1-11.
2. **Milijikovic-Selimovic, B., Dinic, M., Orolovic, J., Babic, T.** 2015. *Staphylococcus aureus*: immunopathogenesis and human immunity. Acta facultatis medicae Naissensis. **32:**243-257.
3. **Radlinski, L., Rowe, S.E., Kartchner, L.B., Maile, R., Cairns, B.A., Vitko, N.P., Gode, C.J., Lachiewicz, A.M. Wolfgang, M.C., Conlon, B.P.** 2017. *Pseudomonas aeruginosa* exoproducts determine antibiotic efficacy against *Staphylococcus aureus*. PLOS Biol. **15:**1-25.
4. **Nguyen, A.T., Jones, J.W., Camara, M., Williams, P., Kane, M.A., Oglesby-Sherrouse, A.G.** 2016. Cystic fibrosis isolates of *Pseudomonas aeruginosa* retain iron-regulated antimicrobial properties against *Staphylococcus aureus* through the action of multiple alkylquinolones. Front. Microbiol. **7:**1171.
5. **Burke, K.A., Lascelles, J.** 1975. Nitrate reductase system in *Staphylococcus aureus* wild type and mutants. J. Bacteriol. **123:**308-316.
6. **Burke, K. A., Lascelles, J.** 1976. Nitrate reductase activity in heme-deficient mutants of *Staphylococcus aureus*. J. Bacteriol. **126:**225-231.

7. **Noto, M.J., Burns, W.J., Beavers, W.N., Skarr, E.P.** 2017. Mechanisms of pyocyanin toxicity and genetic determinants of resistance in *Staphylococcus aureus*. J. Bacteriol. **199:**1-13.

8. **Lei, M.G., Gupta, R.K., Lee, C.Y.** 2017. Proteomics of *Staphylococcus aureus* biofilm matrix in a rat model of orthopedic implant-associated infection. PLOS One. **12:**1-17.

9. **Angus, D.C., van der Poll, T.** 2013. Severe sepsis and septic shock. N. Engl. J. Med. **369:**840-851.

10. **Thakur, S., Thakur, K., Sood, A., Chaudhary, S.** 2016. Bacteriological profile and antibiotic sensitivity pattern of neonatal septicemia in a rural tertiary care hospital in North India. Indian J. Med. Microbiol. **34:** 67-71.

11. **Antonanzas, F., Lozano, C., Torres, C.** 2015. Economic features of antibiotic resistance: the case of methicillin-resistant *Staphylococcus aureus*. PharmacoEconomics. **33:**285-325.

12. **Nichols, C.I., Vose, J.G.** 2016. Clinical outcomes and costs within 90 days of primary or revision total joint arthroplasty. J. Arthroplasty. **31:** 1400-1406.

13. **Macedo-Vinas, M., De Angelis, G., Rohner, P., Safran, E., Stewardson, A., Fankhauser, C., Schrenzel, J., Pittet, D., Harbarth, S.** 2013. Burden of methicillin-resistant *Staphylococcus aureus* infections at a Swiss University hospital: excess length of stay and costs. J. Hosp. Infect. **84:** 132-137.

14. **Jarvis, W.R., Schlosser, J., Chinn, R.Y., Tweeten, S.** 2007. National prevalence of methicillin-resistant *Staphylococcus aureus* in inpatients at US health care facilities, 2006. Am. J. Infect. Control. **35:**631-637.

15. **Cohen, P.R.** 2005. Cutaneous community-acquired methicillin-resistant *Staphylococcus aureus* infection in participants of athletic activities. South. Med. J. **98:**596-602.

16. **Uddin, M.J. Ahn, J.** 2017. Associations between resistance phenotype and gene expression in response to serial exposure to oxacillin and ciprofloxacin in *Staphylococcus aureus*. Lett. Appl. Microbiol. **65:**462-468.

17. **Hasan, R., Acharjee, M., Noor, R.** 2016. Prevalence of vancomycin resistant *Staphylococcus aureus* (VRSA) in methicillin resistant *S. aureus* (MRSA) strains isolated from burn wound infections. Tzu Chi Med. J. **28:**49-53.

18. **Thapaliya, D., Taha, M., Dalman, M.R., Kadariya, J., Smith, T.C.** 2017. Environmental contamination with *Staphylococcus aureus* at a large Midwestern university campus. Sci. Total Environ. **599-600:**1363-1368.

19. **Frozi, J.B., Esper, L.M.R., Franco, R.M.** 2017. Single and multispecies biofilms by *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp. isolated from raw fish and a fish processing unit. Cienc. Rural. **47:**1-6.

20. **Necidova, L., Bagdanovicova, K., Harustiakova, D., Bartova, K.** 2016. Short communication: pasteurization as a means of inactivating staphylococcal enterotoxins A, B, and C in milk. J. Dairy Sci. **99:**8638-8643.

21. **Hu, D.L., Ono, H.K., Isayama, S., Okada, R., Okamura, M., Lei, L.C., Liu, Z.S., Zhang, X.C., Liu, M.Y., Cui, J.C., Nakane, A.** 2017. Biological characteristics of staphylococcal enterotoxin Q and its potential risk for food poisoning. J. Appl. Microbiol. **122:**1672-1679.

22. **Todd, E.C.D.** 1985. Economic loss from foodborne disease outbreaks associated with foodservice establishments. J. Food Prot. **48:**169-180.

23. **Clauditz, A., Resch, A., Wieland, K.P., Peschel, A., Götz, F.** 2006. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. Infect. Immun. **74:**4950-4953.

24. **Mishra, N.N., Liu, G.Y., Yeaman, M.R., Nast, C.C., Proctor, R.A., McKinnell, J., Bayer, A.S.** 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob. Agents Chemother. **55:**526-531.

25. **Ma, W., Zhang, D., Li, G., Liu, J., He, G., Zhang, P., Yang, L., Zhu, H., Xu, N., Liang, S.** 2016. Antibacterial mechanism of daptomycin antibiotic against *Staphylococcus aureus* based on a quantitative bacterial proteome analysis. J. Proteomics. **150:**242-251.

26. **Sanders, E.R.** 2012. Aseptic laboratory techniques: plating methods. J. Vis. Exp. **63:**1-18.

27. **Lin, K., Wang, A.** 2001. UV mutagenesis in *Escherichia coli* K-12: cell survival and mutation frequency of the chromosomal genes lacZ, rpoB, ompF, and ampA. J. Exp. Microbiol. Immunol. **1:**32-46.

28. **Furtado, G.L., Medeiros, A.A.** 1980. Single-disk diffusion testing (Kirby-Bauer) of susceptibility of *Proteus mirabilis* to chloramphenicol: significance of the intermediate category. J. Clin. Microbiol. **12:**550-553.

29. **Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M.** 1966. Antibiotic susceptibility testing by a standardized single disk method. J. Clin. Pathol. **45:**493-496.

30. **Doudney, C.O., Young, C.S.** 1962. Ultraviolet light induced mutation and deoxyribonucleic acid replication in bacteria. Genetics. **47:**1125-1138.

31. **Witkin, E.M.** 1976. Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. Bacteriol. Rev. **40:**869-907.

32. **Chapple, R. M., Inglis, B., Stewart, P.R.** 1992. Lethal and mutational effects of solar and UV radiation on *Staphylococcus aureus*. Arch. Microbiol. **157:**242-248.

33. **Swarupa, V., Sundari, A.R., Prasad, U.V., Yeswanth, S., Sarma, P.V.G.K.** 2013. Induced mutagenesis in *Staphylococcus aureus* ATCC 12600 resulting in over expression of streptomycin 3"-adenyltransferase 1. J. Pure Appl. Microbio. **7:**1-6.

34. **Essers, L., Radebold, K.** 1980. Rapid and reliable identification of *Staphylococcus aureus* by a latex agglutination test. J. Clin. Microbiol. **12:**641-643.

35. **Sorum, M., Skov, R.** 2009. Evaluation of monostaph plus in comparison to two other latex agglutination tests for the identification of *Staphylococcus aureus*. J. Rapid Methods Autom. Microbiol. **17:**414-419.

36. **Myrick, B.A., Ellner, P.D.** 1981. Evaluation of the latex slide agglutination test for identification of *Staphylococcus aureus*. J. Clin. Microbiol. **15:**275-277.

37. **Köck, R., Werner, P., Friedrich, A.W., Fegeler, C., Becker, K.** 2015. Persistence of nasal colonization with human pathogenic bacteria and associated antimicrobial resistance in the German general population. New Microbe and New Infect. **9:**24-34.

38. **Kloos, W.E., Wolfshohl, J.F.** 1982. Identification of *Staphylococcus* species with the API STAPH-IDENT system. J. Clin. Microbiol. **16:**509-516.

39. **Price, J. Gordon, N.C., Crook, D., Llewelyn, M., Paul, J.** 2012. The usefulness of whole genome sequencing in the management of *Staphylococcus aureus* infections. Clin. Microbiol. Infect. **19:**784-789.

40. **Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F. Fierer, J., Nizet, V.** 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J. Exp. Med. **202:**209-215.

41. **Fenton, C., Keating, G.M., Curran, M.P.** 2004. Daptomycin. Drugs. **64:**455-465.

42. **Monteiro, A., Neto, W.R.N., Mendes, A.R.S., Pinto, B.L.S., Silva, L.C.N., Ferreira, G.F.** 2017. The rise of virulence and antibiotic resistance in *Staphylococcus aureus*. Intech Open Science, London

43. **Schaad, H.J., Bento, M., Lew, D.P., Vaudaux, P.** 2006. Evaluation of high-dose daptomycin for therapy of experimental *Staphylococcus aureus* foreign body infection. BMC Infect. Dis. **6:**74.

44. **Antonic, V., Stojadinovic, A., Zhang, B., Izadjoo, M.J., Alavi, M.** 2013. *Pseudomonas aeruginosa* induces pigment production and enhances virulence in a white phenotypic variant of *Staphylococcus aureus*. Infect. Drug. Resist. **6:**175-186.

45. **Biswas, L., Biswas, R., Schlag, M., Bertram, R., Götz, F.** 2009. Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **75:**6910-6912.

46. **Katzif, S., Lee, E.U., Law, A.B., Tzeng, Y.L., Shafer, W.M.** 2005. CspA regulates pigment production in *Staphylococcus aureus* through a sigB-dependent mechanism. J. Bacteriol. **187:**8181-8184.

47. **Kong, C., Neoh, H.M., Nathan, S.** 2016. Targeting *Staphylococcus aureus* toxins: a potential form of anti-virulence therapy. Toxins. **2016:**1-21.

48. **Song, Y., Liu, C.I., Lin, F.Y., No, J.H., Hensler, M. Liu, Y.L., Jheng, W.Y., Low, J., Liu, G.Y., Nizet, V., Wang, A.H.J., Oldfield, E.** 2009. Inhibition of staphyloxanthin virulence factor biosynthesis in *Staphylococcus aureus*: in vitro, in vivo, and crystallographic results. **52:** 3869-3880.

BIOGRAPHY

William Hunter Miears was raised in Kerrville, Texas by two wonderful and supportive parents, Gary and Robin Miears, along with his younger brother, Cole Miears. Hunter graduated from Angelo State University in May 2018 with a Bachelor of Science in biology with minors in chemistry and German language and highest university honors. Hunter was an active member in the Honors Program, the Honors Student Association, and Baptist Student Ministry and was also a member of Tri-Beta Biological Honors Society, Alpha Chi Honors Society, and Gamma Mu Omega Honors Society. He has presented this research at the Tri-Beta South Central Regional Convention in 2017 and 2018, the Tri-Beta National Convention in Monterrey, California in 2018, as well as at the National Collegiate Honors Council Conference in Atlanta, Georgia in 2017 and the Great Plains Honors Council Conference in Stillwater, Oklahoma in 2018.

Hunter served as the Vice President of the Honors Student Association for two years where he coordinated volunteers to accumulate over 6,000 hours of community service as an organization. Additionally, he was a member of the Angelo State Baptist Student Ministry leadership team and served as a member and president of the Baptist Student Ministries State of Texas Leadership Team, as a member of the executive board of the Baptist General Convention of Texas. Following graduation, Hunter will attend Texas Tech University Health Sciences Center School of Medicine in Lubbock, Texas, to begin his training toward becoming a physician.

Hunter can be reached via email at hunter.miears@gmail.com